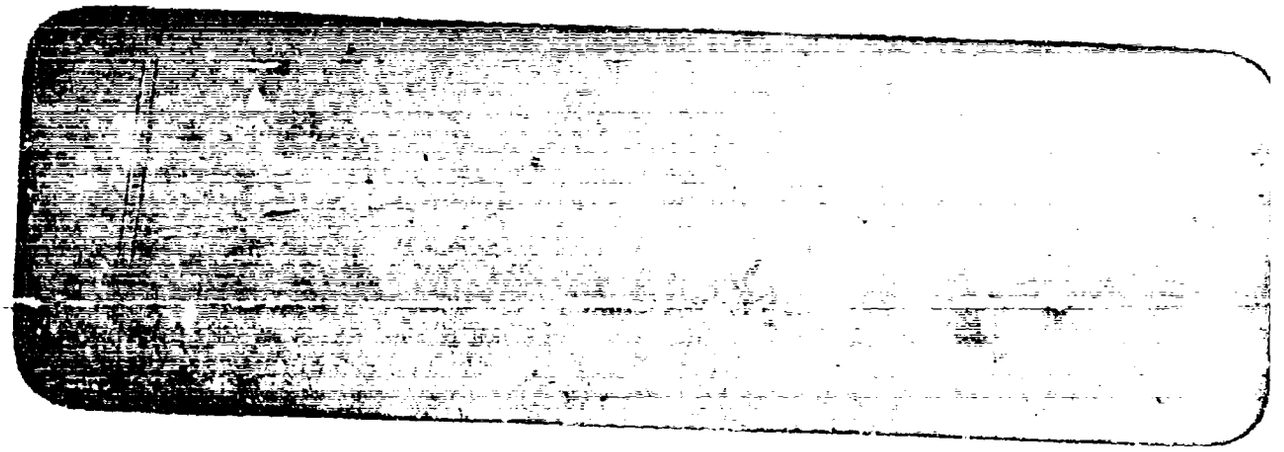

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Amino Acid Composition of Crystalline Botulinum Toxin, Type A

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An amino acid analysis of crystalline botulinum toxin, type A, based on microbiological assay, was reported by this laboratory in 1947 (H. J. Buehler, E. J. Schantz, and C. Lamanna, J. Biol. Chem. **169**:295, 1947). D. Van Alstyne, J. Gerwing, and J. H. Tremaine (J. Bacteriol. **92**: 796, 1966) have recently reported on the amino acid composition of a low molecular weight biologically active component of this toxin and have compared it to the crystalline material. We have reinvestigated the amino acid composition of the crystalline material using the more accurate chromatographic techniques now available. In this note, the results of this analysis are presented, and some of the implications they have upon the subunit structure of the toxin are discussed.

A single batch of crystalline toxin isolated by the method of J. T. Duff et al. (J. Bacteriol. **73**: 42, 1957) was used for all analyses. This preparation was homogeneous in the ultracentrifuge and exhibited a specific activity by mouse intraperitoneal injection of 2.3×10^8 LD_{50} per mg of N. The methods used were described in a previous publication (L. Spero et al., Biochemistry **4**:1024, 1965).

The nitrogen content of the toxin was redetermined and found to be 16.08%. The sulfur content was 0.69%, and phosphorus was <0.05%. (We are grateful to J. F. Alicino of the Squibb Institute for Medical Research for doing the sulfur analysis.) The $E^{1\%}_{1cm}$ at 278 mμ was found to be 16.6. The results of the amino acid analyses are presented in Table 1. Each figure represents the average of three analyses. Each analysis was performed on 24-, 48-, 72-, and 96-hr hydrolysates run singly. The results tend to confirm the earlier analysis. Some of the amino acids, particularly phenylalanine and glycine, show striking differences, but for the most part the errors may be attributed to inherent inaccuracies in the older methodology. In addition, crystalline toxin isolated from a nonsporulating strain has been analyzed with no detectable differences.

It was recognized early by C. Lamanna (Proc. Soc. Exptl. Biol. Med. **69**:332, 1948) that the crystalline material (molecular weight, 900,000) did not represent a single, simple molecule. The first physical evidence of dissociation of the toxin was obtained by J. Wagman and J. B. Bateman (Arch. Biochem. Biophys. **31**:424, 1951). Under conditions of mild alkalinity and high ionic strength, toxic fractions of the order of 150,000 molecular weight were obtained. Material of the same size has now been prepared by a column chromatographic procedure by B. R. DasGupta, D. A. Boroff, and E. Rothstein (Biochem. Biophys. Res. Commun. **22**:750, 1966), and they report a specific toxicity of 1.86×10^8 LD_{50} per mg or about five times that of the parent material.

The possibility of even smaller subunits—in the 10,000 to 20,000 range—in the crystalline material was suggested by one of us (E. J. Schantz, publication 999-FP-1, Public Health Service, Cincinnati, 1964, p. 91), on the basis of diffusion studies in agar gel. Recently, a report from Dolman's laboratory (J. Gerwing, C. E. Dolman, and H. S. Bains, J. Bacteriol. **89**:1383, 1965) described the isolation in good percentage yield of an active botulinum toxin fragment with a molecular weight of 12,000 to 13,000.

It is abundantly clear then that the crystalline toxin is an aggregate of subunits of considerably smaller molecular size. The smallest unit present may well be a component with a molecular weight of 12,000, but it is certain that there are other moieties present in the aggregated macromolecule. Evidence for this comes from at least three avenues of investigation: (i) the presence of a hemagglutinating material in the crystalline toxin (C. Lamanna, Proc. Soc. Exptl. Biol. Med. **69**:332, 1948); (ii) the increased specific activity obtained in one of the isolated fragments (B. R. DasGupta, D. A. Boroff, and E. Rothstein, Biochem. Biophys. Res. Commun. **22**:750, 1966); and (iii) the present amino acid analysis. The smallest repeating unit containing histidine has a molecular weight of 15,400, the smallest containing cystine, 27,400, and the smallest containing cysteine, 26,700.

TABLE 1. *Amino acid composition of botulinum toxin, type A*

Amino acid	Nitrogen (g/100 g of protein)	Amino acid residues (g/100 g of protein)	Amino acid residues (g/100 g of protein) (lit. value)	No. of residues per 10^5 g of protein	No. of residues per 10^5 g of protein (lit. value)
Lysine	1.45	6.65 ± 0.12	6.78	51.9	52.9
Histidine	0.27	0.89 ± 0.02	0.91	6.5	6.6
Arginine	1.32	3.69 ± 0.04	4.14	23.6	26.5
Aspartic acid	2.24	18.41 ± 0.13	17.34	159.9	150.7
Threonine	0.73	5.27 ± 0.12	7.19	52.1	71.1
Serine	0.87	5.42 ± 0.12	3.60	62.2	41.3
Glutamic acid	1.06	9.81 ± 0.10	13.67	76.0	105.9
Proline	0.37	2.58 ± 0.10	2.19	26.6	27.1
Glycine	0.48	1.97 ± 0.03	1.05	34.5	18.4
Alanine	0.46	2.35 ± 0.03	3.12	33.0	43.9
Half-cystine	0.10	0.74[b]	0.45	7.3	4.5
Cysteine	0.05	0.39[c]	0.23	3.8	2.2
Valine	0.61	4.34 ± 0.04	4.45	43.8	44.9
Methionine	0.12	1 ± 0.01	0.93	8.7	7.1
Isoleucine	1.25	8.. ± 0.04	10.33	79.3	91.3
Leucine	1.04	8.40 ± 0.07	8.91	74.2	78.7
Tyrosine	0.76	8.87 ± 0.17	12.18	54.4	74.6
Phenylalanine	0.53	5.59 ± 0.12	1.04	38.0	7.1
Tryptophan	0.37	2.46	1.69	13.2	9.1
Amide groups	1.78	2.03 ± 0.09[d]	2.43	126.8[d]	152.1
Total	15.86	97.95		849.0	

[a] From Table 1 of H. J. Buehler et al. (J. Biol. Chem. 169:295, 1947).
[b] Difference between total cystine and cysteine determined as cysteic acid and cysteine determined directly.
[c] Determined by alkylation with iodocetate in guanidine.
[d] Not included in total.

A comparison of the present analysis with the composition of the low molecular weight toxin of J. Gerwing et al. (J. Bacteriol. 89:1383, 1965) also supports this view. The discrepancies are so significant that the material with a molecular weight of 12,000 to 13,000 can not be the only protein subunit in the crystalline material. Studies intending to elucidate the subunit composition of crystalline botulinum toxin are presently being carried out in this laboratory.